1
2 **GLYCOPROTEIN AND APOLIPOPROTEIN BIOPOLYMER**
MARKERS PREDICTIVE OF ALZHEIMERS DISEASE
3

4 <u>FIELD OF THE INVENTION</u>

5   This invention relates to the field of characterizing

6 the existence of a disease state; particularly to the

7 utilization of mass spectrometry to elucidate particular

8 biopolymer markers indicative or predictive of a particular

9 disease state, and most particularly to specific biopolymer

10 markers whose up-regulation, down-regulation, or relative

11 presence in disease vs. normal states has been determined to

12 be useful in disease state assessment and therapeutic target

13 recognition, development and validation.

14 <u>BACKGROUND OF THE INVENTION</u>

15   Methods utilizing mass spectrometry for the analysis of

16 a target polypeptide have been taught wherein the polypeptide

17 is first solubilized in an appropriate solution or reagent

18 system. The type of solution or reagent system, e.g.,

19 comprising an organic or inorganic solvent, will depend on

20 the properties of the polypeptide and the type of mass

21 spectrometry performed and are well-known in the art (see,

22 e.g., Vorm et al. (1994) Anal. Chem. 66:3281 (for MALDI) and

23 Valaskovic et al. (1995) Anal. Chem. 67:3802 (for ESI). Mass

24 spectrometry of peptides is further disclosed, e.g., in WO

McHale & Slavin, P.A. - Atty. Doc. 2132.096   -1-

Express Mail Label EV 001631516 US

1   93/24834 by Chait et al.

2   In one prior art embodiment, the solvent is chosen so

3   that the risk that the molecules may be decomposed by the

4   energy introduced for the vaporization process is

5   considerably reduced, or even fully excluded. This can be

6   achieved by embedding the sample in a matrix, which can be an

7   organic compound, e.g., sugar, in particular pentose or

8   hexose, but also polysaccharides such as cellulose. These

9   compounds are decomposed thermolytically into CO_2 and H_2O so

10   that no residues are formed which might lead to chemical

11   reactions. The matrix can also be an inorganic compound,

12   e.g., nitrate of ammonium which is decomposed practically

13   without leaving any residues. Use of these and other solvents

14   are further disclosed in U.S. Pat. No. 5,062,935 by Schlag et

15   al.

16   Prior art mass spectrometer formats for use in analyzing

17   the translation products include ionization (I) techniques,

18   including but not limited to matrix assisted laser desorption

19   (MALDI), continuous or pulsed electrospray (ESI) and related

20   methods (e.g., IONSPRAY or THERMOSPRAY), or massive cluster

21   impact (MCI); these ion sources can be matched with detection

22   formats including linear or non-linear reflection time-of-

23   flight (TOF), single or multiple quadropole, single or

1   multiple magnetic sector, Fourier Transform ion cyclotron

2   resonance (FTICR), ion trap, and combinations thereof (e.g.,

3   ion-trap/time-of-flight). For ionization, numerous

4   matrix/wavelength combinations (MALDI) or solvent

5   combinations (ESI) can be employed. Subattomole levels of

6   protein have been detected, for example, using ESI

7   (Valaskovic, G. A. et al., (1996) Science 273:1199-1202) or

8   MALDI (Li, L. et al., (1996) J. Am. Chem. Soc. 118:1662-1663)

9   mass spectrometry.

10   ES mass spectrometry has been introduced by Fenn et al.

11   (J. Phys. Chem. 88, 4451-59 (1984); PCT Application No. WO

12   90/14148) and current applications are summarized in recent

13   review articles (R. D. Smith et al., Anal. Chem. 62, 882-89

14   (1990) and B. Ardrey, Electrospray Mass Spectrometry,

15   *Spectroscopy* Europe, 4, 10-18 (1992)). MALDI-TOF mass

16   spectrometry has been introduced by Hillenkamp et al.

17   ("Matrix Assisted UV-Laser Desorption/Ionization: A New

18   Approach to Mass Spectrometry of Large Biomolecules,"

19   Biological Mass Spectrometry (Burlingame and McCloskey,

20   editors), Elsevier Science Publishers, Amsterdam, pp. 49-60,

21   1990). With ESI, the determination of molecular weights in

22   femtomole amounts of sample is very accurate due to the

23   presence of multiple ion peaks which all could be used for

1   the mass calculation.

2   The mass of the target polypeptide determined by mass

3   spectrometry is then compared to the mass of a reference

4   polypeptide of known identity. In one embodiment, the target

5   polypeptide is a polypeptide containing a number of repeated

6   amino acids directly correlated to the number of

7   trinucleotide repeats transcribed/translated from DNA; from

8   its mass alone the number of repeated trinucleotide repeats

9   in the original DNA which coded it, may be deduced.

10   U.S. Patent No. 6,020,208 utilizes a general category of

11   probe elements (i.e., sample presenting means) with Surfaces

12   Enhanced for Laser Desorption/Ionization (SELDI), within

13   which there are three (3) separate subcategories. The SELDI

14   process is directed toward a sample presenting means (i.e.,

15   probe element surface) with surface-associated (or surface-

16   bound) molecules to promote the attachment (tethering or

17   anchoring) and subsequent detachment of tethered analyte

18   molecules in a light-dependent manner, wherein the said

19   surface molecule(s) are selected from the group consisting of

20   photoactive (photolabile) molecules that participate in the

21   binding (docking, tethering, or crosslinking) of the analyte

22   molecules to the sample presenting means (by covalent

23   attachment mechanisms or otherwise).

1   PCT/EP/04396 teaches a process for determining the

2   status of an organism by peptide measurement. The reference

3   teaches the measurement of peptides in a sample of the

4   organism which contains both high and low molecular weight

5   peptides and acts as an indicator of the organism's status.

6   The reference concentrates on the measurement of low

7   molecular weight peptides, i.e. below 30,000 Daltons, whose

8   distribution serves as a representative cross-section of

9   defined controls. Contrary to the methodology of the instant

10   invention, the '396 patent strives to determine the status of

11   a healthy organism, i.e. a "normal" and then use this as a

12   reference to differentiate disease states. The present

13   inventors do not attempt to develop a reference "normal", but

14   rather strive to specify particular markers whose presence,

15   absence or relative strength/concentration in disease vs.

16   normal is diagnostic of at least one specific disease state

17   or whose up-regulation or down-regulation is predictive of at

18   least one specific disease state, whereby the presence of

19   said marker serves as a positive indicator useful in

20   distinguishing disease state. This leads to a simple method

21   of analysis which can easily be performed by an untrained

22   individual, since there is a positive correlation of data.

23   On the contrary, the '396 patent requires a complicated

1   analysis by a highly trained individual to determine disease

2   state versus the perception of non-disease or normal

3   physiology.

4   Richter et al, Journal of Chromatography B, 726(1999)

5   25-35, refer to a database established from human

6   hemofiltrate comprised of a mass database and a sequence

7   database. The goal of Richter et al was to analyze the

8   composition of the peptide fraction in human blood. Using

9   MALDI-TOF, over 20,000 molecular masses were detected

10   representing an estimated 5,000 different peptides. The

11   conclusion of the study was that the hemofiltrate (HF)

12   represented the peptide composition of plasma. No

13   correlation of peptides with relation to normal and/or

14   disease states is made.

15   As used herein, "analyte" refers to any atom and/or

16   molecule; including their complexes and fragment ions. The

17   term may refer to a single component or a set of components.

18   In the case of biological molecules/macromolecules or

19   "biopolymers", such analytes include but are not limited to:

20   polypeptides, polynucleotides, proteins, peptides,

21   antibodies, DNA, RNA, carbohydrates, steroids, and lipids,

22   and any detectable moiety thereof, e.g. immunologically

23   detectable fragments. Note that most important biomolecules

1   under investigation for their involvement in the structure or

2   regulation of life processes are quite large (typically

3   several thousand times larger than H_2O).

4   As used herein, the term "molecular ions" refers to

5   molecules in the charged or ionized state, typically by the

6   addition or loss of one or more protons (H^+).

7   As used herein, the term "molecular fragmentation" or

8   "fragment ions" refers to breakdown products of analyte

9   molecules caused, for example, during laser-induced

10 desorption (especially in the absence of added matrix).

11   As used herein, the term "solid phase" refers to the

12 condition of being in the solid state, for example, on the

13 probe element surface.

14   As used herein, "gas" or "vapor phase" refers to

15 molecules in the gaseous state (i.e., in vacuo for mass

16 spectrometry).

17   As used herein, the term "analyte desorption/ionization"

18 refers to the transition of analytes from the solid phase to

19 the gas phase as ions. Note that the successful

20 desorption/ionization of large, intact molecular ions by

21 laser desorption is relatively recent (circa 1988)--the big

22 breakthrough was the chance discovery of an appropriate

23 matrix (nicotinic acid).

1   As used herein, the term "gas phase molecular ions"

2   refers to those ions that enter into the gas phase. Note that

3   large molecular mass ions such as proteins (typical

4   mass=60,000 to 70,000 times the mass of a single proton) are

5   typically not volatile (i.e., they do not normally enter into

6   the gas or vapor phase). However, in the procedure of the

7   present invention, large molecular mass ions such as proteins

8   do enter the gas or vapor phase.

9   As used herein in the case of MALDI, the term "matrix"

10   refers to any one of several small, acidic, light absorbing

11   chemicals (e.g., CHCA (alpha-cyano-4-hydroxy-cinnamic acid),

12   nicotinic or sinapinic acid) that is mixed in solution with

13   the analyte in such a manner so that, upon drying on the

14   probe element, the crystalline matrix-embedded analyte

15   molecules are successfully desorbed (by laser irradiation)

16   and ionized from the solid phase (crystals) into the gaseous

17   or vapor phase and accelerated as intact molecular ions. For

18   the MALDI process to be successful, analyte is mixed with a

19   freshly prepared solution of the chemical matrix (e.g.,

20   10,000:1 matrix:analyte) and placed on the inert probe

21   element surface to air dry just before the mass spectrometric

22   analysis. The large fold molar excess of matrix, present at

23   concentrations near saturation, facilitates crystal formation

1   and entrapment of analyte.

2   As used herein, "energy absorbing molecules (EAM)"

3   refers to any one of several small, light absorbing chemicals

4   that, when presented on the surface of a probe, facilitate

5   the neat desorption of molecules from the solid phase (i.e.,

6   surface) into the gaseous or vapor phase for subsequent

7   acceleration as intact molecular ions. The term EAM is

8   preferred, especially in reference to SELDI. Note that

9   analyte desorption by the SELDI process is defined as a

10   surface-dependent process (i.e., neat analyte may be placed

11   on a surface composed of bound EAM or EAM and analyte may be

12   mixed prior to placement on a surface). In contrast, MALDI is

13   presently thought to facilitate analyte desorption by a

14   volcanic eruption-type process that "throws" the entire

15   surface into the gas phase. Furthermore, note that some EAM

16   when used as free chemicals to embed analyte molecules as

17   described for the MALDI process will not work (i.e., they do

18   not promote molecular desorption, thus they are not suitable

19   matrix molecules).

20   As used herein, "probe element" or "sample presenting

21   device" refers to an element having the following properties:

22   it is inert (for example, typically stainless steel) and

23   active (probe elements with surfaces enhanced to contain EAM

1   and/or molecular capture devices).

2   As used herein, "MALDI" refers to Matrix-Assisted Laser

3   Desorption/Ionization.

4   As used herein, "TOF" stands for Time-of-Flight.

5   As used herein, "MS" refers to Mass Spectrometry.

6   As used herein, "MS/MS" refers to multiple sequential

7   mass spectrometry.

8   As used herein "MALDI-TOF MS" refers to Matrix-assisted

9   laser desorption/ionization time-of-flight mass spectrometry.

10   As used herein, "ESI" is an abbreviation for

11   electrospray ionization.

12   As used herein, "chemical bonds" is used simply as an

13   attempt to distinguish a rational, deliberate, and

14   knowledgeable manipulation of known classes of chemical

15   interactions from the poorly defined kind of general

16   adherence observed when one chemical substance (e.g., matrix)

17   is placed on another substance (e.g., an inert probe element

18   surface). Types of defined chemical bonds include

19   electrostatic or ionic (+/-) bonds (e.g., between a

20   positively and negatively charged groups on a protein

21   surface), covalent bonds (very strong or "permanent" bonds

22   resulting from true electron sharing), coordinate covalent

23   bonds (e.g., between electron donor groups in proteins and

1 transition metal ions such as copper or iron), and

2 hydrophobic interactions (such as between two noncharged

3 groups), weak dipole and London force or induced dipole

4 interactions.

5 As used herein, "electron donor groups" refers to the

6 case of biochemistry, where atoms in biomolecules (e.g, N, S,

7 O) "donate" or share electrons with electron poor groups

8 (e.g., Cu ions and other transition metal ions).

9 As used herein, the term "biopolymer markers indicative

10 or predictive of a disease state" is interpreted to mean that

11 a biopolymer marker which is strongly present in a normal

12 individual, but is down-regulated in disease is predictive of

13 said disease; while alternatively, a biopolymer marker which

14 is strongly present in a disease state, but is down-regulated

15 in normal individuals, is indicative of said disease state.

16 Biopolymer markers which are present in both disease and

17 normal states are indicative/predictive based upon their

18 relative strengths in disease vs. normal, along with the

19 observation regarding when their signal strengthens/weakens

20 relative to disease manifestation or progression.

21 As used herein, the term "disease state assessment" is

22 interpreted to mean quantitative or qualitative determination

23 of the presence/absence of the disease, with or without an

1   ability to determine severity, rapidity of onset, or

2   resolution of the disease state, e.g. a return to a normal

3   physiological state.

4   As used herein, the term "therapeutic target

5   recognition, development, and validation" refers to any

6   concept or method which enables an artisan to recognize,

7   develop, or validate the efficacy of a therapeutic moiety

8   which is effected in conjunction with a chemical or physical

9   interaction with one or more of the biopolymer markers of the

10   instant invention.

11   As used herein, the term "polypeptide" is interpreted to

12   mean a polymer composed of amino acid residues, related

13   naturally occurring structural variants, and synthetic non-

14   naturally occurring analogs thereof linked via peptide bonds,

15   related naturally occurring structural variants, and

16   synthetic non-naturally occurring analogs thereof. Synthetic

17   polypeptides can be synthesized, for example, using an

18   automated polypeptide synthesizer. The term "protein"

19   typically refers to large polypeptides. The term "peptide"

20   typically refers to short polypeptides. "Polypeptide(s)"

21   refers to any peptide or protein comprising two or more amino

22   acids joined to each other by peptide bonds or modified

23   peptide bonds. "Polypeptide(s)" refers to both short chains,

1   commonly referred to as peptides, oligopeptides and oligomers

2   and to longer chains generally referred to as proteins.

3   Polypeptides may contain amino acids other than the 20 gene

4   encoded amino acids. "Polypeptide(s)" include those modified

5   either by natural processes, such as processing and other

6   post-translational modifications, but also by chemical

7   modification techniques. Such modifications are well

8   described in basic texts and in more detailed monographs, as

9   well as in a voluminous research literature, and they are

10   well-known to those of skill in the art. It will be

11   appreciated that the same type of modification may be present

12   in the same or varying degree at several sites in a given

13   polypeptide. Also, a given polypeptide may contain many types

14   of modifications. Modifications can occur anywhere in a

15   polypeptide, including the peptide backbone, the amino acid

16   side-chains, and the amino or carboxyl termini. Modifications

17   include, for example, acetylation, acylation, ADP-

18   ribosylation, amidation, covalent attachment of flavin,

19   covalent attachment of a heme moiety, covalent attachment of

20   a nucleotide or nucleotide derivative, covalent attachment of

21   a lipid or lipid derivative, covalent attachment of

22   phosphotidylinositol, cross-linking, cyclization, disulfide

23   bond formation, demethylation, formation of covalent cross-

1   links, formation of cysteine, formation of pyroglutamate,

2   formylation, gamma-carboxylation, glycosylation, GPI anchor

3   formation, hydroxylation, iodination, methylation,

4   myristoylation, oxidation, proteolytic processing,

5   phosphorylation, prenylation, racemization, glycosylation,

6   lipid attachment, sulfation, gamma-carboxylation of glutamic

7   acid residues, hydroxylation and ADP-ribosylation,

8   selenoylation, sulfation, transfer-RNA mediated addition of

9   amino acids to proteins, such as arginylation, and

10   ubiquitination. See, for instance, PROTEINS--STRUCTURE AND

11   MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman

12   and Company, New York (1993) and Wold, F., Posttranslational

13   Protein Modifications: Perspectives and Prospects, pgs. 1-12

14   in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C.

15   Johnson, Ed., Academic Press, New York (1983); Seifter et

16   al., Meth. Enzymol. 182:626-646 (1990) and Rattan et al.,

17   Protein Synthesis: Posttranslational Modifications and Aging,

18   Ann. N.Y. Acad. Sci. 663: 48-62 (1992). Polypeptides may be

19   branched or cyclic, with or without branching. Cyclic,

20   branched and branched circular polypeptides may result from

21   post-translational natural processes and may be made by

22   entirely synthetic methods, as well.

23   As used herein, the term "polynucleotide" is interpreted

1   to mean a polymer composed of nucleotide units.

2   Polynucleotides include naturally occurring nucleic acids,

3   such as deoxyribonucleic acid ("DNA") and ribonucleic acid

4   ("RNA") as well as nucleic acid analogs. Nucleic acid analogs

5   include those which include non-naturally occurring bases,

6   nucleotides that engage in linkages with other nucleotides

7   other than the naturally occurring phosphodiester bond or

8   which include bases attached through linkages other than

9   phosphodiester bonds. Thus, nucleotide analogs include, for

10   example and without limitation, phosphorothioates,

11   phosphorodithioates, phosphorotriesters,

12   phosphoramidates, boranophosphates, methylphosphonates,

13   chiral-methyl phosphonates, 2-O-methyl ribonucleotides,

14   peptide-nucleic acids (PNAs), and the like. Such

15   polynucleotides can be synthesized, for example, using an

16   automated DNA synthesizer. The term "nucleic acid" typically

17   refers to large polynucleotides. The term "oligonucleotide"

18   typically refers to short polynucleotides, generally no

19   greater than about 50 nucleotides. It will be understood that

20   when a nucleotide sequence is represented by a DNA sequence

21   (i.e., A, T, G, C), this also includes an RNA sequence (i.e.,

22   A, U, G, C) in which "U" replaces T.

23   As used herein, the term "detectable moiety" or a

1   "label" refers to a composition detectable by spectroscopic,

2   photochemical, biochemical, immunochemical, or chemical

3   means. For example, useful labels include ^{32}P, ^{35}S,

4   fluorescent dyes, electron-dense reagents, enzymes (e.g., as

5   commonly used in an ELISA), biotin-streptavadin, dioxigenin,

6   haptens and proteins for which antisera or monoclonal

7   antibodies are available, or nucleic acid molecules with a

8   sequence complementary to a target. The detectable moiety

9   often generates a measurable signal, such as a radioactive,

10   chromogenic, or fluorescent signal, that can be used to

11   quantitate the amount of bound detectable moiety in a sample.

12   The detectable moiety can be incorporated in or attached to a

13   primer or probe either covalently, or through ionic, van

14   der Waals or hydrogen bonds, e.g., incorporation of

15   radioactive nucleotides, or biotinylated nucleotides that are

16   recognized by streptavadin. The detectable moiety may be

17   directly or indirectly detectable. Indirect detection can

18   involve the binding of a second directly or indirectly

19   detectable moiety to the detectable moiety. For example, the

20   detectable moiety can be the ligand of a binding partner,

21   such as biotin, which is a binding partner for streptavadin,

22   or a nucleotide sequence, which is the binding partner for a

23   complementary sequence, to which it can specifically

1   hybridize. The binding partner may itself be directly

2   detectable, for example, an antibody may be itself labeled

3   with a fluorescent molecule. The binding partner also may be

4   indirectly detectable, for example, a nucleic acid having a

5   complementary nucleotide sequence can be a part of a branched

6   DNA molecule that is in turn detectable through hybridization

7   with other labeled nucleic acid molecules. (See, e.g., P. D.

8   Fahrlander and A. Klausner, Bio/Technology (1988) 6:1165.)

9   Quantitation of the signal is achieved by, e.g.,

10   scintillation counting, densitometry, or flow cytometry.

11   As used herein, the term "antibody or antibodies"

12   includes polyclonal and monoclonal antibodies of any isotype

13   (IgA, IgG, IgE, IgD, IgM), or an antigen-binding portion

14   thereof, including but not limited to F(ab) and Fv fragments,

15   single chain antibodies, chimeric antibodies, humanized

16   antibodies, and a Fab expression library. "Antibody" refers

17   to a polypeptide ligand substantially encoded by an

18   immunoglobulin gene or immunoglobulin genes, or fragments

19   thereof, which specifically binds and recognizes an epitope

20   (e.g., an antigen). The recognized immunoglobulin --genes

21   include the kappa and lambda light chain constant region

22   genes, the alpha, gamma, delta, epsilon and mu heavy chain

23   constant region genes, and the myriad immunoglobulin variable

1 region genes. Antibodies exist, e.g., as intact

2 immunoglobulins or as a number of well characterized

3 fragments produced by digestion with various peptidases. This

4 includes, e.g., Fab' and F(ab)'$_2$ fragments. The term

5 "antibody," as used herein, also includes antibody fragments

6 either produced by the modification of whole antibodies or

7 those synthesized de novo using recombinant DNA

8 methodologies. It also includes polyclonal antibodies,

9 monoclonal antibodies, chimeric antibodies and humanized

10 antibodies. "Fc" portion of an antibody refers to that

11 portion of an immunoglobulin heavy chain that comprises one

12 or more heavy chain constant region domains, CH, CH$_2$ and CH$_3$,

13 but does not include the heavy chain variable region.

14   As used herein, the term "moieties" refers to an

15 indefinite portion of a sample.

16   A "ligand" is a compound that specifically binds to a

17 target molecule.

18   A "receptor" is a compound or portion of a structure

19 that specifically binds to a ligand.

20   A ligand or a receptor (e.g., an antibody) "specifically

21 binds to" or "is specifically immunoreactive with" a compound

22 analyte when the ligand or receptor functions in a binding

23 reaction which is determinative of the presence of the

1 analyte in a sample of heterogeneous compounds. Thus, under

2 designated assay (e.g., immunoassay) conditions, the ligand

3 or receptor binds preferentially to a particular analyte and

4 does not bind in a significant amount to other compounds

5 present in the sample. For example, a polynucleotide

6 specifically binds under hybridization conditions to an

7 analyte polynucleotide comprising a complementary sequence;

8 an antibody specifically binds under immunoassay conditions

9 to an antigen analyte bearing an epitope against which the

10 antibody was raised; and an adsorbent specifically binds to

11 an analyte under proper elution conditions.

12 As used herein, the term "pharmaceutically effective

13 carrier" refers to any solid or liquid material which may be

14 used in creating formulations that further include active

15 ingredients of the instant invention, e.g. biopolymer markers

16 or therapeutics, for administration to a patient.

17 As used herein, the term "agent" is interpreted to mean

18 a chemical compound, a mixture of chemical compounds, a

19 sample of undetermined composition, a combinatorial small

20 molecule array, a biological macromolecule, a bacteriophage

21 peptide display library, a bacteriophage antibody (e.g.,

22 scFv) display library, a polysome peptide display library, or

23 an extract made from biological materials such as bacteria,

1   plants, fungi, or animal cells or tissues. Suitable

2   techniques involve selection of libraries of recombinant

3   antibodies in phage or similar vectors. See, Huse et al.

4   (1989) Science 246: 1275-1281; and Ward et al. (1989) Nature

5   341: 544-546. The protocol described by Huse is

6   rendered more efficient in combination with phage display

7   technology. See, e.g., Dower et al., WO 91/17271 and

8   McCafferty et al., WO 92/01047.

9   As used herein, the term "isolated" is interpreted to

10   mean altered "by the hand of man" from its natural state,

11   i.e., if it occurs in nature, it has been changed or removed

12   from its original environment, or both. For example, a

13   polynucleotide or a polypeptide naturally present in a living

14   organism is not "isolated," but the same polynucleotide or

15   polypeptide separated from the coexisting materials of its

16   natural state is "isolated", as the term is employed herein.

17   As used herein, the term "variant" is interpreted to

18   mean a polynucleotide or polypeptide that differs from a

19   reference polynucleotide or polypeptide respectively, but

20   retains essential properties. A typical variant of a

21   polynucleotide differs in nucleotide sequence from another,

22   reference polynucleotide. Changes in the nucleotide sequence

23   of the variant may or may not alter the amino acid sequence

1   of a polypeptide encoded by the reference polynucleotide.

2   Nucleotide changes may result in amino acid substitutions,

3   additions, deletions, fusions and truncations in the

4   polypeptide encoded by the reference sequence, as discussed

5   below. A typical variant of a polypeptide differs in amino

6   acid sequence from another, reference polypeptide. Generally,

7   differences are limited so that the sequences of the

8   reference polypeptide and the variant are closely similar

9   overall and, in many regions, identical. A variant and

10   reference polypeptide may differ in amino acid sequence by

11   one or more substitutions, additions, deletions in any

12   combination. A substituted or inserted amino acid residue may

13   or may not be one encoded by the genetic code. A variant of a

14   polynucleotide or polypeptide may be a naturally occurring

15   such as an allelic variant, or it may be a variant that is

16   not known to occur naturally. Non-naturally occurring

17   variants of polynucleotides and polypeptides may be made by

18   mutagenesis techniques, by direct synthesis, and by other

19   recombinant methods known to skilled artisans.

20   As used herein, the term "biopolymer marker" refers to a

21   polymer of biological origin, e.g. polypeptides,

22   polynucleotides, polysaccharides or polyglycerides (e.g., di-

23   or tri-glycerides), and may include any fragment, e.g.

1 immunologically reactive fragments, variants or moieties

2 thereof.

3 　　As used herein, the term "fragment" refers to the

4 products of the chemical, enzymatic, or physical breakdown of

5 an analyte. Fragments may be in a neutral or ionic state.

6 　　As used herein, the term "therapeutic avenues" is

7 interpreted to mean any agents, modalities, synthesized

8 compounds, etc., which interact with a biopolymer marker in

9 any manner that facilitates a therapeutic benefit, including

10 immunotherapeutic intervention, e.g. modalities such as

11 administration of an immunologically reactive moiety capable

12 of altering the course, progression and/or manifestation of

13 the disease, as a result of interfering with the disease

14 manifestation process, for example, at the early stages

15 focused upon by the identification of the disease, such as by

16 supplying a moiety capable of modifying the pathogenicity of

17 lymphocytes specific for the biopolymer marker or related

18 components.

19 　　As used herein, the term "interacting with a biopolymer

20 marker" includes any process by which a biopolymer marker may

21 physically or chemically relate with an organism,

22 particularly when this interaction results in the development

23 of therapeutic avenues or in modulation of the disease state.

1   As used herein, the term "therapeutic targets" may thus

2   be defined as those analytes which are capable of exerting a

3   modulating force, wherein "modulation" is defined as an

4   alteration in function inclusive of activity, synthesis,

5   production, and circulating levels. Thus, modulation effects

6   the level or physiological activity of at least one

7   particular disease related biopolymer marker or any compound

8   or biomolecule whose presence, level or activity is linked

9   either directly or indirectly, to an alteration of the

10   presence, level, activity or generic function of the

11   biopolymer marker, and may include pharmaceutical agents,

12   biomolecules that bind to the biopolymer markers, or

13   biomolecules or complexes to which the biopolymer markers

14   bind. The binding of the biopolymer markers and the

15   therapeutic moiety may result in activation (agonist),

16   inhibition (antagonist), or an increase or decrease in

17   activity or production (modulator) of the biopolymer markers

18   or the bound moiety. Examples of such therapeutic moieties

19   include, but are not limited to, antibodies,

20   oligonucleotides, proteins (e.g., receptors), RNA, DNA,

21   enzymes, peptides or small molecules. With regard to

22   immunotherapeutic moieties, such a moiety may be defined as

23   an effective analog for a major epitope peptide which has the

1   ability to reduce the pathogenicity of key lymphocytes which

2   are specific for the native epitope. An analog is defined as

3   having structural similarity but not identity in peptide

4   sequencing able to be recognized by T-cells spontaneously

5   arising and targeting the endogeneous self epitope. A

6   critical function of this analog is an altered T-cell

7   activation which leads to T-cell anergy or death.

8   With the advent of mass spectrometric methods such as

9   MALDI and SELDI and ESI, researchers have begun to utilize a

10   tool that holds the promise of uncovering countless

11   biopolymers which result from translation, transcription and

12   post-translational transcription of proteins from the entire

13   genome.

14   Operating upon the principles of retentate

15   chromatography, SELDI MS involves the adsorption of proteins,

16   based upon their physico-chemical properties at a given pH

17   and salt concentration, followed by selectively desorbing

18   proteins from the surface by varying pH, salt, or organic

19   solvent concentration. After selective desorption, the

20   proteins retained on the SELDI surface, the "chip", can be

21   analyzed using the CIPHERGEN protein detection system, or an

22   equivalent thereof. Retentate chromatography is limited,

23   however, by the fact that if unfractionated body fluids, e.g.

1   blood, blood products, urine, saliva, cerebrospinal fluid,

2   luymph and the like, along with tissue samples, are applied

3   to the adsorbent surfaces, the biopolymers present in the

4   greatest abundance will compete for all the available binding

5   sites and thereby prevent or preclude less abundant

6   biopolymers from interacting with them, thereby reducing or

7   eliminating the diversity of biopolymers which are readily

8   ascertainable.

9   If a process could be devised for maximizing the

10   diversity of biopolymers discernable from a sample, the

11   ability of researchers to accurately determine the relevance

12   of such biopolymers with relation to one or more disease

13   states would be immeasurably enhanced.

14

15   SUMMARY OF THE INVENTION

16   The instant invention is characterized by the use of a

17   combination of preparatory steps, e.g. chromatography and 1-D

18   tricine polyacrylamide gel electrophoresis. Subsequent to

19   which the gel is stained, e.g. with Coomasie blue, silver or

20   rubidium. Next, bands are selected from the gels for further

21   study. Tryptic digestion of each band follows, concluding

22   with the extraction of tryptic peptides from the digest.

23   This extraction may be accomplished utilizing C18 ZIPTIPs, or

1   organic extract and dry technique followed by MALDI Qq TOF

2   (Maldi Quadrupole Quadrupole Time of Flight) processing.

3   Additional methodologies may include SELDI MS, 2-D gel

4   technology, MALDI MS/MS and time-of-flight detection

5   procedures to maximize the diversity of biopolymers which are

6   verifiable within a particular sample. The cohort of

7   biopolymers verified within a sample is then compared to

8   develop data indicating their presence, absence or relative

9   strength/concentration in disease vs normal controls, and

10   further studied to determine whether the up-regulation or

11   down-regulation of a single biopolymer or group of

12   biopolymers is indicative of a disease state or predictive of

13   the development of said disease state. Additionally,

14   biopolymers recognized as being indicative or predictive of a

15   disease state in accordance with the instant invention are

16   useful in therapeutic intervention, e.g. as therapeutic

17   modalities in their own right, in the course of therapeutic

18   target recognition, in the development and validation of

19   efficacious therapeutic modalities, e.g when interrogating or

20   developing phage display libraries, and as ligands or

21   receptors for use in conjunction with therapeutic

22   intervention.

23   Although all manner of biomarkers related to all disease

1   conditions are deemed to be within the purview of the instant

2   invention and methodology, particular significance was given

3   to those markers and diseases associated with the complement

4   system, cognitive diseases, e.g. Alzheimer's disease and

5   Syndrome X and diseases related thereto.

6   The complement system is an important part of non-clonal

7   or innate immunity that collaborates with acquired immunity

8   to destroy invading pathogens and to facilitate the clearance

9   of immune complexes from the system. This system is the

10   major effector of the humoral branch of the immune system,

11   consisting of nearly 30 serum and membrane proteins. The

12   proteins and glycoproteins composing the complement system

13   are synthesized largely by liver hepatocytes. Activation of

14   the complement system involves a sequential enzyme cascade in

15   which the proenzyme product of one step becomes the enzyme

16   catalyst of the next step. Complement activation can occur

17   via two pathways: the classical and the alternative. The

18   classical pathway is commonly initiated by the formation of

19   soluble antigen-antibody complexes or by the binding of

20   antibody to antigen on a suitable target, such as a bacterial

21   cell. The alternative pathway is generally initiated by

22   various cell-surface constituents that are foreign to the

23   host. Each complement component is designated by numerals

1 (C1-C9), by letter symbols, or by trivial names. After a

2 component is activated, the peptide fragments are denoted by

3 small letters. The complement fragments interact with one

4 another to form functional complexes. Ultimately, foreign

5 cells are destroyed through the process of a membrane-attack

6 complex mediated lysis.

7   The C4 component of the complement system is involved in

8 the classical activation pathway. It is a glycoprotein

9 containing three polypeptide chains (α, β, and γ). C4 is a

10 substrate of component C1s and is activated when C1s

11 hydrolyzes a small fragment (C4a) from the amino terminus of

12 the α chain, exposing a binding site on the larger fragment

13 (C4b).

14   The native C3 component consists of two polypeptide

15 chains, α and β. As a serum protein, C3 is involved in the

16 alternative pathway. Serum C3, which contains an unstable

17 thioester bond, is subject to slow spontaneous hydrolysis

18 into C3a and C3b. The C3f component is involved in the

19 regulation required of the complement system which confines

20 the reaction to designated targets. During the regulation

21 process, C3b is cleaved into two parts: C3bi and C3f. C3bi

22 is a membrane-bound intermediate wherein C3f is a free

23 diffusible (soluble) component.

1   Complement components have been implicated in the

2   pathogenesis of several disease conditions. C3 deficiencies

3   have the most severe clinical manifestations, such as

4   recurrent bacterial infections and immune-complex diseases,

5   reflecting the central role of C3. The rapid profusion of

6   C3f moieties and resultant "accidental" lysis of normal cells

7   mediated thereby gives rise to a host of auto-immune

8   reactions. The ability to understand and control these

9   mechanisms, along with their attendant consequences, will

10 enable practitioners to develop both diagnostic and

11 therapeutic avenues by which to thwart these maladies.

12 In the course of defining a plurality of disease

13 specific marker sequences, special significance was given to

14 markers which were evidentiary of a particular disease state

15 or with conditions associated with Syndrome-X. Syndrome-X is

16 a multifaceted syndrome, which occurs frequently in the

17 general population. A large segment of the adult population

18 of industrialized countries develops this metabolic syndrome,

19 produced by genetic, hormonal and lifestyle factors such as

20 obesity, physical inactivity and certain nutrient excesses.

21 This disease is characterized by the clustering of insulin

22 resistance and hyperinsulinemia, and is often associated with

23 dyslipidemia (atherogenic plasma lipid profile), essential

1 hypertension, abdominal (visceral) obesity, glucose

2 intolerance or noninsulin-dependent diabetes mellitus and an

3 increased risk of cardiovascular events. Abnormalities of

4 blood coagulation (higher plasminogen activator inhibitor

5 type I and fibrinogen levels), hyperuricemia and

6 microalbuminuria have also been found in metabolic syndrome-

7 X.

8   The instant inventors view the Syndrome X continuum in

9 its cardiovascular light, while acknowledging its important

10 metabolic component. The first stage of Syndrome X consists

11 of insulin resistance, abnormal blood lipids (cholesterol,

12 triglycerides and free fatty acids), obesity, and high blood

13 pressure (hypertension). Any one of these four first stage

14 conditions signals the start of Syndrome X.

15   Each first stage Syndrome X condition risks leading to

16 another. For example, increased insulin production is

17 associated with high blood fat levels, high blood pressure,

18 and obesity. Furthermore, the effects of the first stage

19 conditions are additive; an increase in the number of

20 conditions causes an increase in the risk of developing more

21 serious diseases on the Syndrome X continuum.

22   A patient who begins the Syndrome X continuum risks

23 spiraling into a maze of increasingly deadly diseases. The

1   next stages of the Syndrome X continuum lead to overt

2   diabetes, kidney failure, and heart failure, with the

3   possibility of stroke and heart attack at any time. Syndrome

4   X is a dangerous continuum, and preventative medicine is the

5   best defense. Diseases are currently most easily diagnosed

6   in their later stages, but controlling them at a late stage

7   is extremely difficult. Disease prevention is much more

8   effective at an earlier stage.

9   In a further contemplated embodiment of the invention,

10   samples may be taken from a patient at one point in time, as

11   a single sample or as multiple samples, or at different

12   points in time such that analysis is carried out on multiple

13   samples for ongoing analysis. Typically, a first sample is

14   taken from a patient upon presentation with possible symptoms

15   of a disease and analyzed according to the invention.

16   Subsequently, some period of time after presentation, for

17   example, about 3 - 6 months after the first presentation, a

18   second sample is taken and analyzed according to the

19   invention. The data can be used, by way of example, to

20   diagnose or monitor a disease state, determine risk

21   assessment, identify therapeutic avenues, or determine the

22   therapeutic value of an agent such as a pharmaceutical.

23   Subsequent to the isolation of particular disease state

1   marker sequences as taught by the instant invention, the

2   promulgation of various forms of risk assessment tests are

3   contemplated which will allow physicians to identify

4   asymptomatic patients before they suffer an irreversible

5   event such as diabetes, kidney failure, and heart failure,

6   and enable effective disease management and preventative

7   medicine. Additionally, the specific diagnostic tests which

8   evolve from this methodology provide a tool for rapidly and

9   accurately diagnosing acute Syndrome X events such as heart

10   attack and stroke, and facilitate treatment.

11   More particularly, biopolymer markers elucidated via

12   methodologies of the instant invention find utility related

13   to broad areas of disease therapeutics. Such therapeutic

14   avenues include, but are not limited to:

15   1)   utilization and recognition of said biopolymer

16   markers, variants or moieties thereof as direct therapeutic

17   modalities, either alone or in conjunction with an effective

18   amount of a pharmaceutically effective carrier;

19   2)   validation of therapeutic modalities or disease

20   preventative agents as a function of biopolymer marker

21   presence or concentration;

22   3)   treatment or prevention of a disease state by

23   formation of disease intervention modalities; e.g. formation

1   of biopolymer/ligand conjugates which intervene at receptor

2   sites to prevent, delay or reverse a disease process;

3   4)   use of biopolymer markers or moieties thereof as a

4   means of elucidating therapeutically viable agents, e.g. from

5   a bacteriophage peptide display library, a bacteriophage

6   antibody library or the like;

7   5)   instigation of a therapeutic immunological

8   response; and

9   6)   synthesis of molecular structures related to said

10   biopolymer markers, moieties or variants thereof which are

11   constructed and arranged to therapeutically intervene in the

12   disease process.

13   A process for identifying or developing therapeutic

14   avenues related to a disease state utilizing any of the above

15   examples may follow results obtained from conducting an

16   analysis inclusive of interacting with a biopolymer including

17   the sequence of the particular disease specific marker or at

18   least one analyte thereof of the present invention. Such

19   treatment or prevention of a disease state by formation of

20   disease intervention modalities may be by the formation of

21   biopolymer/ligand conjugates which intervene at receptor

22   sites to prevent, delay, or reverse a disease process. In

23   addition, a means of elucidating therapeutically viable

1   agents may include the use of a bacteriophage peptide display

2   library or a bacteriophage antibody library. The therapeutic

3   avenues may regulate the presence or absence of the

4   biopolymer including the sequence of the particular disease

5   specific marker or at least one analyte thereof in the

6   present invention.

7   Accordingly, it is an objective of the instant invention

8   to define a disease specific biopolymer marker sequence which

9   is useful in evidencing and categorizing at least one

10   particular disease state.

11   It is an additional objective of the instant invention

12   to develop methods and means of disease therapy, including

13   but not limited to:

14   1) utilization and recognition of said biopolymer

15   markers, variants or moieties thereof as direct therapeutic

16   modalities, either alone or in conjunction with an effective

17   amount of a pharmaceutically effective carrier;

18   2) validation of therapeutic modalities or disease

19   preventative agents as a function of biopolymer marker

20   presence or concentration;

21   3) treatment or prevention of a disease state by

22   formation of disease intervention modalities; e.g. formation

23   of biopolymer/ligand conjugates which intervene at receptor

1   sites to prevent, delay or reverse a disease process;

2   4)   use of biopolymer markers or moieties thereof as a

3   means of elucidating therapeutically viable agents, e.g. from

4   a bacteriophage peptide display library, a bacteriophage

5   antibody library or the like;

6   5)   instigation of a therapeutic immunological

7   response; and

8   6)   synthesis of molecular structures related to said

9   biopolymer markers, moieties or variants thereof which are

10   constructed and arranged to therapeutically intervene in the

11   disease process, e.g. by directly determining the three-

12   dimensional structure of said biopolymer marker directly from

13   an amino acid sequence thereof.

14   It is another objective of the instant invention to

15   evaluate samples containing a plurality of biopolymers for

16   the presence of disease specific biopolymer marker sequences

17   (disease specific markers) which evidence a link to at least

18   one specific disease state.

19   It is a further objective of the instant invention to

20   elucidate essentially all biopolymeric markers, moieties or

21   variants thereof contained within said samples, whereby

22   particularly significant moieties may be identified.

23   It is a further objective of the instant invention

1   provide at least one purified antibody which is specific to

2   said disease specific marker sequence.

3   It is yet another objective of the instant invention to

4   teach a monoclonal antibody which is specific to said disease

5   specific marker sequence.

6   It is a still further objective of the invention to

7   teach polyclonal antibodies raised against said disease

8   specific marker.

9   It is yet an additional objective of the instant

10   invention to teach a diagnostic kit for determining the

11   presence, concentration, or relative strength/concentration

12   of said disease specific marker.

13   It is a still further objective of the instant invention

14   to teach methods for characterizing disease state based upon

15   the identification of said disease specific marker.

16   Other objects and advantages of this invention will

17   become apparent from the following description taken in

18   conjunction with the accompanying drawings wherein are set

19   forth, by way of illustration and example, certain

20   embodiments of this invention. The drawings constitute a

21   part of this specification and include exemplary embodiments

22   of the present invention and illustrate various objects and

23   features thereof.

1

2   BRIEF DESCRIPTION OF THE FIGURES

3   Figure 1 is a photograph of a tricine gel DEAE 3 (Elution)

4   comparing Alzheimers disease versus Age Matched Control;

5   Figure 2 is a trypsin digested spectra graph depicting the

6   ion 1873;

7   Figure 3 is a photograph of a tricine gel Butyl Sepharose 1

8   (Elution) comparing Alzheimers disease versus Age Matched

9   Control;

10 Figure 4 is a trypsin digested spectra graph depicting the

11 ion 1873;

12 Figure 5 is a photograph of a tricine gel HiS 1 (scrub)

13 comparing Alzheimers disease versus Age Matched Control; and

14 Figure 6 is a trypsin digested spectra graph depicting the

15 ion 1394.

16

17 DETAILED DESCRIPTION OF THE INVENTION

18   In earlier work, for example in U.S. Patent application

19 09/846330 filed April 30, 2000, the contents of which is

20 herein incorporated by reference, raw sera was obtained and

21 mixed with formic acid and extracted the peptides with C18

22 reversed phase ZIPTIPs.

23   In the instantly disclosed invention, we deal with

1 proteins generally having a molecular weight of about 20 kD

2 or more. In general, proteins of greater than 20 kD can

3 reliably be fragmented by trypsin or other enzymes. The

4 instant technology incorporates sufficient sensitivity to

5 deal with even the low production of peptides from proteins

6 less than 20 kD clipped from gel.

7 Proteins differ from peptides in that they cannot be

8 effectively resolved by time of flight MS and they are too

9 large (>3kD) to be effectively fragmented by collision with

10 gases. The most commonly used solution to these problems is

11 to resolve the proteins by polyacrylamide gel electrophoresis

12 followed by staining with silver, or coomasie brilliant blue

13 or rubidium dyes or counter staining with Zinc-SDS complexes.

14 Once the proteins have been resolved and visualized with

15 stains the proteins that differ between disease states can

16 then be excised from the gel and the protein purified in the

17 1-D gel band or 2-D gel spot can be cleaved into fragments

18 less that 3 kD by proteolytic enzymes. Once protein has been

19 resolved by gel and cleaved by enzymes, the protein is

20 considered in the form of peptides and therefore can be dealt

21 with as per earlier work (09/846330). The peptide is either

22 collected and purified with C18 reversed phase chromatography

23 or by some other form of chromatography prior to reversed

1   phase separation. The peptide can also be collected in

2   ammonium carbonate buffer that is subsequently evolved by

3   reaction with acid or by removal in organic solvents.

4   Once the peptides are collected they can be sequenced,

5   e.g. with a MALDI-Qq-TOF but also with a TOF-TOF, and

6   ESI-Q-TOF or an ION-TRAP. Other types of MS analysis which

7   may be employed are SELDI MS and MS/MS. The peptides are

8   fragments of the original protein. The peptides are

9   sequenced by fragmentation to produced a spectrum composed of

10   the parts of the peptide. The peptide fragments can be

11   produced by a strong ionization energy with a laser,

12   temperature, electron capture, collision between the peptides

13   themselves or with other objects such as gas molecules. The

14   spacing in terms of mass between the parts of the peptides is

15   a fragmentation pattern. The fragmentation pattern of each

16   peptide from the starting mass to the last remaining amino

17   acid (from either end) is unique.

18   The human genome contains the genes that encode all

19   proteins. The proteolytic cut sites within all these

20   proteins can be predicted from the translated amino acid

21   sequence. The mass of the peptides that result from the

22   predicting cut sites can be calculated. Similarly, the

23   fragmentation pattern from each hypothetical peptide can be

1   predicted. Thus, we can conceptually digest the proteins

2   within the human proteome and fragment them.

3   When a peptide has been "sequenced" it is understood

4   that the peptide fragment has been purified by one of the

5   methods above, i.e. Time of flight (TOF) or by

6   chromatography, before fragmenting it with gas to produce the

7   peptide fragments. The original peptide mass and

8   fragmentation pattern obtained is then fit to those from the

9   theoretical digestion and fragmentation of the genome. The

10   peptide that best matches the theoretical peptides and

11   fragments and is biologically possible, i.e. a potential

12   human blood-borne protein, is thus identified. It is possible

13   to identify plural targets in this fashion.

14

15   Following are exemplary, but non-limiting examples of

16   preparatory protocols useful in the process of the instant

17   invention.

18

19   Preparatory Protocols:

20   Any of these protocols may be selected from a column

21   flow-through stream, a column elution stream, or a column

22   scrub stream.

23   Hi Q is a strong anion exchanger made of methyl acrylate

1 co-polymer with the functional group: $-N^+(CH_3)_2$;

2 Hi S is a strong cation exchanger made of methyl acrylate

3 co-polymer with the functional group: $-SO_3^-$;

4 DEAE is diethylaminoethyl which is a weak cation exchanger

5 made of methyl acrylate co-polymer with the functional group

6 $-N^+(C_2H_5)_2$;

7 PS is phenyl sepharose;

8 BS is butyl sepharose.

9   Note that the supports, i.e. methyl acrylate and

10 sepharose are different, but non-limiting examples, as the

11 same functional group on different supports will function,

12 albeit possibly with different effects.

13 DEAE Column Protocol:

14   1) Cast 200 µl of 50% slurry;

15   2) Equilibrate column in 5 bed volumes of 50 mM

16   tricine pH 8.8 (binding buffer);

17   3) Dissolve 25 µl of sera in 475 µl of binding buffer;

18   4) Wash column in 5 bed volumes of binding buffer;

19   5) Elute column in 120 µl of 0.4 M Phosphate buffer

20   (PB) pH 6.1;

21   6) Elute column in 120 µl of 50 mM citrate buffer

22   pH 4.2;

23   7) Scrub column with 120 µl sequentially with each

1   of 0.1% triton, 1.0% triton and 2% SDS in

2   62.5 mM Tris pH 6.8.

3

4   Butyl Sepharose Column Protocol:

5   1) Cast 150 µl bed volume column;

6   2) Equilibrate column in 5 bed volumes of 1.7 M

7            $(NH_4)_2SO_4$ in 50 mM PB pH 7.0 (binding buffer);

8   3) Dissolve 35 µl of sera in 465 µl of binding buffer

9   and apply;

10   4) Wash column in 5 bed volumes of binding buffer;

11   5) Elute column in 120 µl of 0.4 M $(NH_4)_2SO_4$ in

12   50 mM PB pH 7.0;

13   6) Elute column in 120 µl of 50 mM PB pH 7.0;

14   7) Scrub column with 120 µl sequentially with each

15   of 0.1% triton, 1.0% triton and 2% SDS in

16   62.5 mM Tris pH 6.8.

17

18

19   Phenyl Sepharose Column Protocol:

20   1) Cast 150 µl bed volume column;

21   2) Equilibrate column in 5 bed volumes of

22   1.7 M $(NH_4)_2SO_4$ in 50 mM PB pH 7.0 (binding buffer);

23   3) Dissolve 35 µl of sera in 465 µl of binding

1   buffer and apply;

2   4) Wash column in 5 bed volumes of binding buffer;

3   5) Elute column in 120 µl of 0.2 M $(NH_4)_2SO_4$ in

4   50 mM PB pH 7.0;

5   6) Elute column in 120 µl of 50 mM PB pH 7.0;

6   7) Scrub column with 120 µl sequentially with each

7   of 0.1% triton, 1.0% triton and 2% SDS in

8   62.5 mM Tris pH 6.8.

9

10   HiQ Anion Exchange Mini Column Protocol:

11   1) Dilute sera in sample/running buffer;

12   2) Add HiQ resin to column and remove any air bubbles;

13   3) Add ultrafiltered (UF) water to aid in column

14   packing;

15   4) Add sample/running buffer to equilibrate column;

16   5) Add diluted sera;

17   6) Collect all the flow-through fraction in Eppendorf

18   tubes until level is at resin;

19   7) Add sample/running buffer to wash column;

20   8) Add elution buffer and collect elution in Eppendorf

21   tubes.

22

23   HiS Cation Exchange Mini Column Protocol:

1 1)Dilute sera in sample/running buffer;

2 2)Add HiS resin to column and remove any air bubbles;

3 3)Add UF water to aid in column packing;

4 4)Add sample/running buffer to equilibrate column for

5 sample loading;

6 5)Add diluted sera to column;

7 6)Collect all flow through fractions in Eppendorf

8 tubes until level is at resin;

9 7)Add sample/running buffer to wash column;

10 8)Add elution buffer and collect elution in Eppendorf

11 tubes.

12 Illustrative of the various buffering compositions

13 useful in this technique are:

14 Sample/Running buffers: including but not limited to

15 Bicine buffers of various molarities, pH's, NaCl content,

16 Bis-Tris buffers of various molarities, pH's, NaCl

17 content, Diethanolamine of various molarities, pH's, NaCl

18 content, Diethylamine of various molarities, pH's, NaCl

19 content, Imidazole of various molarities, pH's, NaCl

20 content, Tricine of various molarities, pH's, NaCl

21 content, Triethanolamine of various molarities, pH's, NaCl

22 content, Tris of various molarities, pH's, NaCl content.

23 Elution Buffer: Acetic acid of various molarities, pH's,

1   NaCl content, Citric acid of various molarities, pH's,

2   NaCl content, HEPES of various molarities, pH's, NaCl

3   content, MES of various molarities, pH's, NaCl content,

4   MOPS of various molarities, pH's, NaCl content, PIPES of

5   various molarities, pH's, NaCl content, Lactic acid of

6   various molarities, pH's, NaCl content, Phosphate of

7   various molarities, pH's, NaCl content, Tricine of various

8   molarities, pH's, NaCl content.

9   Following tryptic digestion, additional processing

10   may be carried out, for example:

11   Utilizing a type of micro-chromatographic column called a

12   C18- ZIPTIP available from the Millipore company, the

13   following preparatory steps were conducted.

14   1. Dilute sera in sample buffer

15   2. Aspirate and dispense ZIPTIP in 50% Acetonitrile

16   3. Aspirate and dispense ZIPTIP in Equilibration solution

17   4. Aspirate and dispense in serum sample

18   5. Aspirate and dispense ZIPTIP in Wash solution

19   6. Aspirate and dispense ZIPTIP in Elution Solution

20   Illustrative of the various buffering compositions

21   useful in the present invention are:

22   Sample Buffers (various low pH's): Hydrochloric acid

23   (HCl), Formic acid, Trifluoroacetic acid (TFA),

1 Equilibration Buffers (various low pH's): HCl, Formic

2 acid, TFA;

3 Wash Buffers (various low pH's): HCl, Formic acid, TFA;

4 Elution Solutions (various low pH's and % Solvents):

5 HCl, Formic acid, TFA;

6 Solvents: Ethanol, Methanol, Acetonitrile.

7 Spotting was then performed, for example upon a Gold Chip

8 in the following manner:

9   1. Spot 2 ul of sample onto each spot

10   2. Let sample partially dry

11   As a result of these procedures, the disease specific

12 markers (J02908) apolipoprotein J precursor having a

13 molecular weight of about 1873.9911 daltons and a sequence

14 of (K)LFSDSPITVTVPVEVSR(K); (M74816) sulfated glycoprotein-2

SEQ ID NO:1 [handwritten]

15 having a molecular weight of about 1873.9911 daltons and a

SEQ ID NO:2 [handwritten]

16 sequence of (K)LFDSDPITVTVPVEVSR(K); and (J02908)

17 apolipoprotein J precursor having a molecular weight of

SEQ ID NO:3 [handwritten]

18 about 1393.6963 daltons and a sequence of (R)ASSIIDELFQDR(F)

19 related to Alzheimers disease were found.

20 Figures 1, 3 and 5 are photographs of a gel which is

21 indicative of the presence/absence of the marker in disease

22 vs. control and, in cases where the marker is always

23 present, the relative strength, e.g. the up or down

1 regulation of the marker relative to categorization of

2 disease state is deduced.

3 A method for evidencing and categorizing at least one

4 disease state is disclosed. The steps taken include

5 obtaining a sample from a patient, preferably human, and

6 conducting MS analysis on the sample. As a result, at least

7 one biopolymer marker sequence or analyte thereof is

8 isolated from the sample which undergoes evidencing and

9 categorizing and is compared to the biopolymer marker

10 sequence as disclosed in the present invention. The step of

11 evidencing and categorizing is particularly directed to

12 biopolymer markers or analytes thereof linked to at least

13 one risk of disease development of the patient or related to

14 the existence of a particular disease state.

15 In addition, various kits are contemplated for use by

16 the present invention. One such kit provides for

17 determining the presence of the disease specific biopolymer

18 marker. At least one biochemical material is incorporated

19 which is capable of specifically binding with a biomolecule

20 which includes at least the disease specific biopolymer

21 marker or analyte thereof, and a means for determining

22 binding between the biochemical material and the

23 biomolecule. The biochemical material for any of the

1   contemplated kits, by way of example an antibody or at least

2   one monoclonal antibody specific therefore, or biomolecule

3   may be immobilized on a solid support and include at least

4   one labeled biochemical material which is preferably an

5   antibody. The sample utilized for any of the kits may be a

6   fractionated or unfractionated body fluid or a tissue

7   sample. Non-limiting examples of such fluids are blood,

8   blood products, urine, saliva, cerebrospinal fluid, and

9   lymph.

10   Further contemplated is a kit for diagnosing,

11   determining risk-assessment, and identifying therapeutic

12   avenues related to a disease state. This kit includes at

13   least one biochemical material which is capable of

14   specifically binding with a biomolecule which includes at

15   least one biopolymer marker including the sequence of the

16   particular disease specific biopolymer marker or an

17   analyte thereof related to the disease state. Also

18   included is a means for determining binding between the

19   biochemical material and the biomolecule, whereby at least

20   one analysis to determine a presence of a marker, analyte

21   thereof, or a biochemical material specific thereto, is

22   carried out on a sample. As previously described,

23   analysis may be carried out on a single sample or multiple

1   samples.

2   In accordance with various stated objectives of the

3   invention, the skilled artisan, in possession of the

4   specific disease specific marker as instantly disclosed,

5   would readily carry out known techniques in order to raise

6   purified biochemical materials, e.g. monoclonal and/or

7   polyclonal antibodies, which are useful in the production of

8   methods and devices useful as point-of-care rapid assay

9   diagnostic or risk assessment devices as are known in the

10   art.

11   The specific disease markers which are analyzed

12   according to the method of the invention are released into

13   the circulation and may be present in the blood or in any

14   blood product, for example plasma, serum, cytolyzed blood,

15   e.g. by treatment with hypotonic buffer or detergents and

16   dilutions and preparations thereof, and other body fluids,

17   e.g. CSF, saliva, urine, lymph, and the like. The

18   presence of each marker is determined using antibodies

19   specific for each of the markers and detecting specific

20   binding of each antibody to its respective marker. Any

21   suitable direct or indirect assay method may be used to

22   determine the level of each of the specific markers

23   measured according to the invention. The assays may be

1   competitive assays, sandwich assays, and the label may be

2   selected from the group of well-known labels such as

3   radioimmunoassay, fluorescent or chemiluminescence

4   immunoassay, or immunoPCR technology. Extensive discussion

5   of the known immunoassay techniques is not required here

6   since these are known to those of skilled in the art. See

7   Takahashi et al. (Clin Chem 1999;45(8):1307) for a

8   detailed example of an assay.

9   A monoclonal antibody specific against the disease

10   marker sequence isolated by the present invention may be

11   produced, for example, by the polyethylene glycol (PEG)

12   mediated cell fusion method, in a manner well-known in the

13   art.

14   Traditionally, monoclonal antibodies have been made

15   according to fundamental principles laid down by Kohler

16   and Milstein. Mice are immunized with antigens, with or

17   without, adjuvants. The splenocytes are harvested from

18   the spleen for fusion with immortalized hybridoma

19   partners. These are seeded into microtiter plates where

20   they can secrete antibodies into the supernatant that is

21   used for cell culture. To select from the hybridomas that

22   have been plated for the ones that produce antibodies of

23   interest, the hybridoma supernatants are usually tested

1   for antibody binding to antigens in an ELISA (enzyme

2   linked immunosorbent assay) assay. The idea is that the

3   wells that contain the hybridoma of interest will contain

4   antibodies that will bind most avidly to the test antigen,

5   usually the immunizing antigen. These wells are then

6   subcloned in limiting dilution fashion to produce

7   monoclonal hybridomas. The selection for the clones of

8   interest is repeated using an ELISA assay to test for

9   antibody binding. Therefore, the principle that has been

10   propagated is that in the production of monoclonal

11   antibodies the hybridomas that produce the most avidly

12   binding antibodies are the ones that are selected from

13   among all the hybridomas that were initially produced.

14   That is to say, the preferred antibody is the one with

15   highest affinity for the antigen of interest.

16   There have been many modifications of this procedure

17   such as using whole cells for immunization. In this

18   method, instead of using purified antigens, entire cells

19   are used for immunization. Another modification is the

20   use of cellular ELISA for screening. In this method

21   instead of using purified antigens as the target in the

22   ELISA, fixed cells are used. In addition to ELISA tests,

23   complement mediated cytotoxicity assays have also been

1   used in the screening process. However, antibody-binding

2   assays were used in conjunction with cytotoxicity tests.

3   Thus, despite many modifications, the process of producing

4   monoclonal antibodies relies on antibody binding to the

5   test antigen as an endpoint.

6   The purified monoclonal antibody is utilized for

7   immunochemical studies.

8   Polyclonal antibody production and purification

9   utilizing one or more animal hosts in a manner well-known

10   in the art can be performed by a skilled artisan.

11   Another objective of the present invention is to

12   provide reagents for use in diagnostic assays for the

13   detection of the particularly isolated disease specific

14   marker sequences of the present invention.

15   In one mode of this embodiment, the marker sequences

16   of the present invention may be used as antigens in

17   immunoassays for the detection of those individuals

18   suffering from the disease known to be evidenced by said

19   marker sequence. Such assays may include but are not

20   limited to: radioimmunoassay, enzyme-linked immunosorbent

21   assay (ELISA), "sandwich" assays, precipitin reactions,

22   gel diffusion immunodiffusion assay, agglutination assay,

23   fluorescent immunoassays, protein A or G immunoassays and

1   immunoelectrophoresis assays.

2   According to the present invention, monoclonal or

3   polyclonal antibodies produced against the disease

4   specific marker sequence of the instant invention are

5   useful in an immunoassay on samples of blood or blood

6   products such as serum, plasma or the like, cerebrospinal

7   fluid or other body fluid, e.g. saliva, urine, lymph, and

8   the like, to diagnose patients with the characteristic

9   disease state linked to said marker sequence. The

10   antibodies can be used in any type of immunoassay. This

11   includes both the two-site sandwich assay and the single

12   site immunoassay of the non-competitive type, as well as

13   in traditional competitive binding assays.

14   Particularly preferred, for ease and simplicity of

15   detection, and its quantitative nature, is the sandwich or

16   double antibody assay of which a number of variations

17   exist, all of which are contemplated by the present

18   invention. For example, in a typical sandwich assay,

19   unlabeled antibody is immobilized on a solid phase, e.g.

20   microtiter plate, and the sample to be tested is added.

21   After a certain period of incubation to allow formation of

22   an antibody-antigen complex, a second antibody, labeled

23   with a reporter molecule capable of inducing a detectable

1   signal, is added and incubation is continued to allow

2   sufficient time for binding with the antigen at a

3   different site, resulting with a formation of a complex of

4   antibody-antigen-labeled antibody. The presence of the

5   antigen is determined by observation of a signal which may

6   be quantitated by comparison with control samples

7   containing known amounts of antigen.

8   Antibodies may also be utilized against the disease

9   specific markers, as haptens, to create an antibody

10   response against the protein to which it binds, thereby

11   identifying targets for treatment of the disease or a sub-

12   class thereof.

13   Lastly, the markers and associated antibodies provide

14   a tool for monitoring the progress of a patient during a

15   therapeutic treatment, so as to determine the usefulness

16   of a novel therapeutic agent.

17   All patents and publications mentioned in this

18   specification are indicative of the levels of those

19   skilled in the art to which the invention pertains. All

20   patents and publications are herein incorporated by

21   reference to the same extent as if each individual

22   publication was specifically and individually indicated to

23   be incorporated by reference.

1   It is to be understood that while a certain form of

2 the invention is illustrated, it is not to be limited to

3 the specific form or arrangement herein described and

4 shown. It will be apparent to those skilled in the art

5 that various changes may be made without departing from

6 the scope of the invention and the invention is not to be

7 considered limited to what is shown and described in the

8 specification and drawings/figures.

9   One skilled in the art will readily appreciate that

10 the present invention is well adapted to carry out the

11 objectives and obtain the ends and advantages mentioned,

12 as well as those inherent therein. The oligonucleotides,

13 peptides, polypeptides, biologically related compounds,

14 methods, procedures and techniques described herein are

15 presently representative of the preferred embodiments, are

16 intended to be exemplary and are not intended as

17 limitations on the scope. Changes therein and other uses

18 will occur to those skilled in the art which are

19 encompassed within the spirit of the invention and are

20 defined by the scope of the appended claims. Although the

21 invention has been described in connection with specific

22 preferred embodiments, it should be understood that the

23 invention as claimed should not be unduly limited to such

1   specific embodiments. Indeed, various modifications of the

2   described modes for carrying out the invention which are

3   obvious to those skilled in the art are intended to be

4   within the scope of the following claims.

5